

Klebsiella to *Salmonella* gene transfer within rumen protozoa: Implications for antibiotic resistance and rumen defaunation[☆]

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Abstract

The rumen has long been thought to be a site of gene transfer for microorganisms. Rumen protozoa (RPz) are active predators of bacteria that can harbor antibiotic resistance genes. In this study, RPz were assessed as sites of gene transfer between two bacterial species, *Salmonella* and *Klebsiella*. One *Klebsiella* isolate carried a plasmid bearing *bla*_{CMY-2}, encoding an extended-spectrum β -lactamase conferring ceftriaxone resistance, while the *Salmonella* was susceptible to ceftriaxone yet capable of thriving within protozoa. In vitro studies revealed that ceftriaxone-resistant *Salmonella* could be isolated following co-incubation of *Salmonella* and *Klebsiella* with RPz obtained from adult cattle and goats. Ceftriaxone-resistant *Salmonella* were not recovered in the presence of an inhibitor of protozoa engulfment or when a protozoa-sensitive *Salmonella* was part of the co-incubation. This transfer event was additionally observed in vitro for protozoa-independent stressors although at a significantly lower frequency. The gene transfer event was related to bacterial conjugation since a conjugation inhibitor, nalidixic acid, perturbed the phenomenon. Ceftriaxone-resistant *Salmonella* were recovered from calves, sheep, and goats co-challenged with ceftriaxone-resistant *Klebsiella* and ceftriaxone-sensitive *Salmonella*. However, the transfer event was not observed in calves and sheep that were defaunated prior to the co-challenge. Moreover, *Salmonella* transconjugants were isolated from separate bovine in vivo studies involving a *Klebsiella* donor carrying a plasmid conferring colicin activity while no such transconjugants were obtained from defaunated calves. These results provide an important basis for evaluating and preventing the spread of antibiotic resistance and other selective advantages for pathogens present in ruminants.

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Keywords: *Salmonella*; Conjugation; Protozoa; *Klebsiella pneumoniae*; Rumen; Antibiotic resistance; Ceftriaxone

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1. Introduction

The microbial community of ruminant animals represents a diverse mix of prokaryotic and eukaryotic organisms interacting in complex metabolic pathways, representing a novel nutritional strategy for their animal hosts (Russell and Rychlik, 2001). In addition to these biochemical associations, there is opportunity for exchange at the genetic level. Gene transfer in the rumen is relevant given the mounting spread of antibiotic resistant bacteria and the public health implications for veterinary antimicrobial therapies. The first documentation of the transfer of antibiotic resistance between bacteria species in the rumen was reported over 25 years ago in sheep (Smith, 1975, 1977). Subsequent work has offered indirect evidence for gene transfer in ruminants, focusing either on the ability of rumen bacteria to acquire antibiotic resistance (Barbosa et al., 1999; Jonecova et al., 1994; Scott et al., 1997) or on the promotion of gene exchange by factors in the rumen environment (Fliegerova, 1993; Mizan et al., 2002; Scott and Flint, 1995). However, in vivo studies regarding pathogenic bacteria are lacking and the parameters and mechanism(s) of this phenomenon remain largely unexplored.

To date, the direct role of rumen protozoa (RPz) in antibiotic resistance gene transfer between bacteria has not been addressed. While in vitro studies have shown that engulfment by aquatic protozoa enhances bacterial conjugation (Schlimme et al., 1997), studies on the fate of bacteria in RPz are lacking. Differential uptake, as well as survival, of bacteria species by various RPz was initially described in a series of studies by G.S. Coleman (summarized in Dehority, 2003; Williams and Coleman, 1992) though it has generally been presumed that bacteria ingested by RPz succumb to digestive processes in protozoal vacuoles (Dehority, 2003). However, multiple studies have shown that *Salmonella* can survive within protozoa (Gaze et al., 2003; King et al., 1988; Tezcan-Merdol et al., 2004) including RPz (Rasmussen et al., 2005), which is notable given the potential for acquisition of antibiotic resistance genes in the rumen environment.

Herein, we investigated the role of RPz in bacterial gene transfer in the rumen. We identified and characterized the transfer of ceftriaxone resistance

from a ceftriaxone-resistant *Klebsiella* isolate to a ceftriaxone-sensitive *Salmonella* recipient in the rumen. Co-incubations of these bacteria were conducted in the presence of RPz in vitro (bovine, caprine) and in vivo (bovine, caprine, and ovine). In order to determine whether the transfer event occurred independent of RPz, in vitro studies were conducted using disabled RPz and in vivo studies were conducted using defaunated animals. Experiments were also conducted in the absence of RPz in order to investigate the extent of the transfer event in conditions that were independent of RPz. Additional experiments examined the transfer of a colicin-encoding plasmid from *Klebsiella* to *Salmonella* in vivo.

2. Materials and methods

2.1. Strains used

All *Salmonella* strains (Table 1) were obtained from frozen stock cultures at the National Animal Disease Center (Ames, IA). For isolation and identification of the recipient *Salmonella* strains, GFP expression was conferred with the previously described pGFP plasmid containing a zeocin (Invitrogen) resistance marker (Frana and Carlson, 2001). *K. pneumoniae*, herein designated as TCR2003, and *K. ornithinolytica* (designated as TME2003) were isolated from turtle fecal samples collected in Iowa in 2003 and were identified using the BBL CRYSTAL Enteric/Nonfermenter ID System (Becton, Dickinson and Company, Sparks, MD) typing method. Ceftriaxone resistance in TCR2003 was examined by determining the MIC for ceftriaxone (breakpoint 64 µg/ml; Sigma) as per Clinical and Laboratory Standards Institute standards. Colicin activity, i.e., anti-*Escherichia coli* activity, in TME2003 was demonstrated using disk diffusion overlay and a zone of inhibition as described previously (Carlson et al., 2001) with TME2003 serving as the test strain and *E. coli* as the indicator strain. A colicin was putatively verified as the bacteriocidal agent by growing *E. coli* overnight in the presence of filter-sterilized TME2003 supernatant with or without the protease trypsin; there was no *E. coli* growth in the absence of trypsin (data not shown). TME2003 was also found to be

Table 1
Summary of strains and plasmids used in this study

Strain, plasmid, or cell line	Relevant characteristics	Reference
<i>Salmonella enterica</i> serotype Typhimurium SL1344	Antibiotic sensitive	Wray and Sojka (1978)
<i>S. Typhimurium</i> χ 4232	Nalidixic acid resistance	Courtesy of Dr. Roy Curtiss
<i>S. enterica</i> serotype Panama	Unable to survive in an intracellular environment (e.g., RPz)	Rasmussen et al. (2005)
<i>Klebsiella pneumoniae</i> TCR2003 ^a	Isolated from turtle feces recovered from Iowa; ceftriaxone-resistant	This study
<i>K. ornithinolytica</i> TME2003 ^a	Isolated from turtle feces recovered from Iowa; ampicillin-resistant; colicin activity	This study
pGFP	GFP expression plasmid, zeocin and kanamycin resistance	Frana and Carlson (2001)
BHK-21	Non-phagocytic, fibroblasts	ATCC
BoMac	Phagocytic, macrophages	Stabel and Stabel (1995)
HEp-2	Non-phagocytic, epithelial cells	ATCC
VERO	Non-phagocytic, epithelial cells	ATCC

^a Identified by BBL crystal typing.

ampicillin-resistant (breakpoint 32 μ g/ml; Sigma), which has been shown to be linked to colicin activity (Perez-Diaz and Clowes, 1980).

2.2. In vitro protozoa experiments

Approximately 100 ml of post-prandial rumen fluid was removed from a non-lactating Jersey cow or a non-lactating female goat fed standard hay and grain diets. Fluid was removed through a rumen fistula that was surgically introduced previously, filtered with cheesecloth, and mixed with an equal volume of Coleman's buffer D (Coleman and Reynolds, 1982). After settling for 2 h under CO₂ at 39 °C, RPz were aspirated under CO₂, and washed twice with approximately 45 ml Coleman's buffer D then centrifuged for 20 s at 230 \times g. Pelleted RPz were resuspended in 30 ml Coleman's buffer D under CO₂. Approximately 10⁹ CFU of both *S. enterica* serotype Typhimurium SL1344 (Wray and Sojka, 1978) (except where noted) and *K. pneumoniae* TCR2003 were incubated with approximately 10⁶ RPz in sealed glass tubes overnight on a rocker at 37 °C. In order to assess whether the transfer of ceftriaxone resistance occurred within protozoa, two controls were used. Cytochalasin D (5 μ g/ml; Sigma), which disrupts the RPz cytoskeleton (Moffat and Tompkins, 1992) and thus prevents engulfment, was used as a control in incubations with both bovine and caprine protozoa. An isolate of *S. enterica* serotype Panama, which does

not survive inside macrophages and thus does not survive protozoal engulfment (Rasmussen et al., 2005), was also used as an additional recipient control strain. In all experiments, protozoa were lysed using a bead beater for 60 s and approximately 1 ml was centrifuged and resuspended in 200 μ l Lennox L broth (GIBCO-BRL). Next, 50 μ l was spread on Lennox L agar (GIBCO-BRL) plates containing ceftriaxone (32 μ g/ml; Sigma). Plates were incubated at 37 °C overnight and colonies were enumerated the next day.

2.3. Mammalian cell line inoculations

BHK-21 cells (ATCC) were maintained in high glucose DMEM (GIBCO-BRL) with sodium bicarbonate, pH 7, and 10% fetal bovine serum (Sigma). Bovine macrophages (BoMac) (Stabel and Stabel, 1995) were maintained in RPMI 1640 (GIBCO-BRL) with 10% fetal bovine serum. HEp-2 cells (ATCC) were maintained in RPMI 1640 with 10% fetal bovine serum and 100 μ g/ml gentamicin (Sigma). VERO cells (ATCC) were maintained in RPMI 1640 with 5% fetal bovine serum and 1% penicillin/streptomycin (Sigma). All cells were incubated at 37 °C in a 5% CO₂ humidified environment. Approximately 10⁹ CFU of SL1344 and TCR2003 were added to confluent cells in fresh media and incubated for 48 h. At that point, the tissue culture cells were detached from the flask, disrupted using a bead beater, and the material recovered was plated as described above for

the protozoa. If putative transconjugants were isolated, a repeat experiment was conducted using RPz-sensitive *S. Panama* as a recipient strain to determine if the transfer event occurred within the eukaryotic cell. That is, the isolation of a ceftriaxone-resistant *S. Panama* would suggest that the transfer event occurred extracellularly.

2.4. Stress experiments

In order to assess whether stress could promote conjugation, i.e., an SOS-type response, co-incubations were performed under inhibitory conditions for *Klebsiella*. Approximately 10^9 CFU of SL1344 and TCR2003 were added to 5 ml Lennox L broth and incubated aerobically overnight at 37 °C in the presence of 4% ethanol, 50 mM NaCl, pH 4.4, or 25 µg/ml zeocin. The latter was used as a stressor for TCR2003 since SL1344 is zeocin resistant via pGFP. A fifth culture was grown overnight at 42 °C and an additional culture was grown with no treatment. For each culture, transfer of ceftriaxone resistance to SL1344 was assessed by plating 50 µl aliquots on Lennox L agar ($n = 6$) containing ceftriaxone.

2.5. Assessment of conjugation

TCR2003 and *S. Typhimurium* χ 4232 (nalidixic acid resistant; courtesy of Dr. Roy Curtiss) were grown in the presence of nalidixic acid (50 µg/ml; Sigma), a known inhibitor of conjugation (Provence and Curtiss, 1994). Gene exchange was then assessed in the presence of zeocin or caprine protozoa.

2.6. In vivo experiments

Conjugative transfer of ceftriaxone resistance to *Salmonella* was assessed in vivo in three ruminant species: bovine (Holstein or Jersey calves, 30–50 kg), caprine (approximately 80 kg), and ovine (approximately 80 kg) subjects. In addition, conjugative transfer of colicin activity to *Salmonella* was assessed in separate in vivo bovine experiments. Animals were orally challenged with donor and recipient strains as described previously (Carlson et al., 2002). Animals were euthanized 24–36 h after administration of bacteria. Fluid was removed from the abomasum and 1 ml was inoculated into a series of *Salmonella*-

selective media (Wood and Rose, 1992) containing ceftriaxone, when TCR2003 was the donor, or ampicillin when TME2003 was the donor. Specifically, *Salmonella* were selected for by inoculating abomasal fluid into GN broth (Difco) followed by Rappaport-Vassiliadis R10 broth (Difco). Next, cultures were transferred to Lennox L broth containing either ceftriaxone or ampicillin, followed by plating on BG Sulfa agar (Difco) plates containing the respective antibiotic. In order to assess the role of protozoa in the gene transfer event, calves and adult sheep were defaunated using two consecutive daily oral doses of 0.7 mg/kg dioctyl sodium sulphosuccinate (DSS), preceded by 24 h fasting, as described previously (Orpin, 1977). Control animals were administered water on the same dosing and fasting schedule. Upon necropsy, rumen fluid was also sampled in order to evaluate the efficacy of defaunation. Rumen fluid was filtered through cheesecloth and added to an equal volume of Coleman's buffer D. Three 1 ml aliquots of settled material were microscopically examined (10 \times) at 1 and 12 h after collection for enumeration of RPz using a hemocytometer. All in vivo treatments were performed in triplicate. Animal experiments were approved by the Animal Care and Use Committee at the National Animal Disease Center (protocol 3462).

2.7. Characterization of isolates

Individual colonies recovered from ceftriaxone plates from each of the above experiments were grown overnight in Lennox L broth with ceftriaxone. MICs for ceftriaxone were then measured using two-fold serial dilutions as per Clinical and Laboratory Standards Institute standards. Cultures with visible growth (i.e., OD₆₀₀ > 0.3) were identified as *Salmonella* spp. with PCR, using boiled cell lysates as templates, targeting the *sipB*–*sipC* junction (Carlson et al., 1999). Reaction conditions for all PCRs in this study were as follows: 95 °C for 5 min, followed by 25 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Reactions included 2.5 mM MgCl₂, 1 \times PCR buffer, 0.2 mM deoxynucleoside triphosphates, 2.5 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 1 µM primers, and 2 µl boiled cell lysate for template in a total volume of 50 µl.

Table 2
Nucleotide sequences and uses of oligonucleotide primers used in this study

Primer designation	Sequence (5' → 3')	Use	Reference
<i>sipB</i> – <i>C</i>	F: ACAGCAAAATGCGGATGCTT; R: GCGCGCTCAGTGTAGGACTC	Identify <i>S. Typhimurium</i>	Carlson et al. (1999)
ampC	F: ATGATGAAAAAATCGTTATGC; R: TTGCAGCTTTTCAAGAATGCGC	Identify β -lactamase	Koeck et al. (1997)
ampCi	F: GCATAACGATTTTTTCATCAT; R: GCGCATCTTGAAAAGCTACAA	Sequence plasmid	This study
ampCi2	F: TAGCAAGAGATGACCCACCG; R: GGATCCTTTACCGACCC	Sequence plasmid	This study
CandD	F: CTTATTGCTCAAAAGGGCACT; R: ATTTTCCACCCACTGTGATG	Distinguish plasmid “C” and “D” from “B”	This study
IS26	F: GGCACGTGTGCAAAGTTAGC; R: GTCGGTGGTGATAAATTATCAT	Distinguish plasmid “C” from “D”	This study

2.8. Determination of the basis for ceftriaxone resistance

The identity of the β -lactamase gene transferred to the *S. Typhimurium* recipient was determined using PCR with the consensus primers, ampCF and ampCR, that are capable of amplifying *bla*_{LAT-1}, *bla*_{CMY-2}, *bla*_{CMY-4}, and *bla*_{BIL-1} (Koeck et al., 1997) (Table 2). Plasmid DNA was isolated using the large construct kit (Qiagen, Valencia, CA) as per manufacturer's specifications. The amplicon resulting from the in vivo caprine experiment was purified using the Wizard DNA Clean-up System (Promega, Madison, WI) and sequenced by the Iowa State University DNA Sequencing and Synthesis Facility using the ampCF and ampCR primers. In order to gain information on the identity of the plasmid, complementary versions of these PCR primers were used for sequencing reactions with the corresponding large construct prep as template (Table 2). Additional primers were designed based on resulting sequence for subsequent sequencing reactions (Table 2). Sequences were subject to BLAST searches and additional PCRs were used to differentiate between three previously described plasmids (“B”, “C”, and “D”; Giles et al., 2004) with high identity to the sequence data submitted (>97%). To distinguish plasmids “C” and “D” from “B”, primers were designed to amplify the “C and D” specific region (Table 2). To distinguish plasmid “C”, which contains IS26, from “D”, an IS26 PCR was used (Table 2).

2.9. Characterization of colicin activity

Ampicillin-resistant isolates were obtained from abomasal fluid and identified as *Salmonella* using the *sipB*–*C* PCR as described above. Colicin activity was evaluated as described previously (Carlson et al., 2001). Briefly, disks (Bacto concentration disks, sterile blanks; Difco) were soaked in liquid culture (approximately 10⁹ CFU/ml) and added to a lawn of *E. coli* on Lennox L agar plates, then incubated overnight and assessed for a zone of inhibition. For the negative control, pre-conjugation SL1344 was used as the test strain.

3. Results

3.1. In vitro transfer of ceftriaxone resistance in RPz and other eukaryotic cells

Initial in vitro incubations containing the donor TCR2003 (ceftriaxone-resistant), the recipient SL1344 (ceftriaxone-sensitive), and bovine RPz yielded ceftriaxone-resistant SL1344 (Table 3). The same results were obtained with caprine RPz. When protozoa engulfment was disabled with cytochalasin, no ceftriaxone-sensitive SL1344 were isolated. No putative transconjugants were obtained when *S. Panama*, which is not capable of survival in macrophages or RPz (Rasmussen et al., 2005), was used as the recipient strain.

Because it was possible that uptake by any eukaryotic cell could precipitate the plasmid transfer

Table 3

Summary of *Klebsiella*–*Salmonella* gene transfer events observed in vitro. *K. pneumoniae* TCR2003 was used as the donor strain in all studies

Recipient	Condition	Replicates	Total number of ceftriaxone-resistant <i>Salmonella</i> recovered	Average frequency of transfer event ^a
SL1344	Bovine RPz	8	6185	1.8×10^{-5}
SL1344	Bovine RPz + cytochalasin D (5 µg/ml)	4	0	–
SL1344	Caprine RPz	4	1078	6.7×10^{-6}
SL1344	Caprine RPz + cytochalasin D (5 µg/ml)	4	0	–
SL1344	4% ethanol	8	$\sim 8 \times 10^6$	~ 0.25
SL1344	Salt (50 mM NaCl)	8	0	–
SL1344	pH 4	8	0	–
SL1344	Zeocin (25 µg/ml)	8	$\sim 8 \times 10^6$	~ 0.25
SL1344	Temperature (42 °C)	8	0	–
SL1344	No treatment	8	0	–
SL1344	BHK-21	12	1	1.9×10^{-9}
SL1344	BoMac	12	84 ($n = 4$); \sim 8×10^6 ($n = 8$)	2.8×10^{-7} ($n = 4$); \sim 1.5×10^{-2} ($n = 8$)
SL1344	HEp-2	4	0	–
SL1344	VERO	12	1	4.6×10^{-9}
χ4232	Zeocin (25 µg/ml)	4	$\sim 4 \times 10^6$	~ 0.25
χ4232	Zeocin + nalidixic acid (50 µg/ml)	4	0	–
χ4232	Caprine RPz	4	818	3.0×10^{-6}
χ4232	Caprine RPz + nalidixic acid	4	0	–
<i>S. panama</i>	Caprine RPz	4	0	–
<i>S. panama</i>	BoMac	8	0	–

^a Average frequency of transfer equals average number of ceftriaxone-resistant *Salmonella* standardized for 10^9 potential recipients.

event, the donor and recipient strains were instead incubated with mammalian tissue culture cells. Only incubations with BoMacs, cells with engulfment and bacteriocidal activities (Stabel and Stabel, 1995) similar to those found in RPz, yielded ceftriaxone-resistant SL1344 (Table 3). *S. Panama* was then used as the recipient strain in BoMac incubations and no ceftriaxone-resistant *S. Panama* transconjugants were recovered.

To investigate the role of stress in the donation of the plasmid, SL1344 and TCR2003 were grown under a series of growth-limiting conditions. No ceftriaxone-resistant SL1344 were obtained from normal condition incubations or from growth in low pH, high salt, and high temperature but ceftriaxone-resistant isolates were generated from growth in 4% ethanol (Table 3).

3.2. Assessment of conjugation as the mode of gene transfer

Conjugation was assessed as the mechanism of plasmid transfer between *S. Typhimurium* and TCR2003 by using nalidixic acid, an inhibitor of conjugation (Provence and Curtiss, 1994). *S. Typhimurium* χ4232, a nalidixic acid resistant strain, was used as the recipient for in vitro co-incubations with TCR2003 in the presence of zeocin or caprine protozoa. Ceftriaxone-resistant χ4232 were only recovered in the absence of nalidixic acid (Table 3).

3.3. In vivo gene transfer experiments

In vivo experiments were conducted in bovine, caprine, and ovine subjects. For all three types of

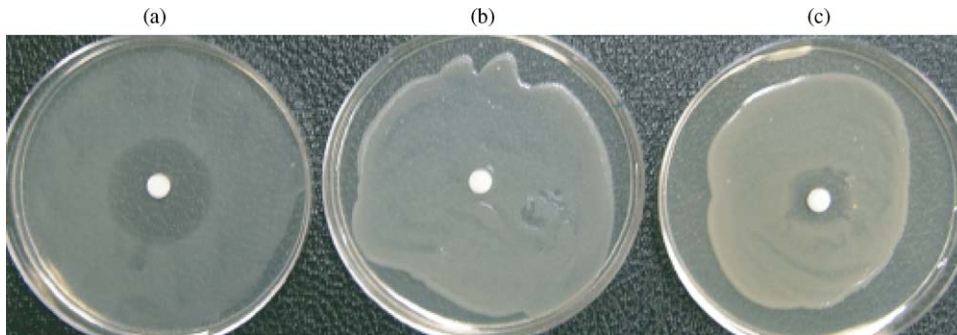


Fig. 1. Demonstration of colicin activity in transconjugant *Salmonella* Typhimurium SL1344. Disks soaked in the isolates were placed on a lawn of *Escherichia coli*. Test bacteria include: (a) *Klebsiella ornithinolytica* TME2003; (b) native *S. Typhimurium* SL1344; (c) in vivo calf isolate of SL1344 (transconjugant). The differing zone sizes between (a) and (c) are likely due to differing levels of colicin expression.

ruminant, ceftriaxone-resistant SL1344 were recovered from abomasal fluid. No ceftriaxone-resistant SL1344 were obtained from calves or sheep defaunated with DSS. For the colicin gene transfer experiment in calves, ampicillin-resistant SL1344 were recovered from abomasal fluid and no isolates were obtained from defaunated calves. These in vivo isolates of SL1344 demonstrated colicin activity like that observed for the TME2003 donor strain (Fig. 1).

Microscopic examination of rumen contents in the defaunation studies confirmed an absence of RPz in animals treated with DSS. RPz counts for untreated sheep were $3.1 \times 10^5 \pm 3.7 \times 10^5$ protozoa/ml ($n = 3$) while $7.3 \times 10^4 \pm .3 \times 10^4$ protozoa/ml ($n = 3$) were

recovered from untreated calves. Defaunation of goats was not performed due to the narrow therapeutic index of DSS in goats as determined empirically.

3.4. Characterization of the *Salmonella* transconjugants and the ceftriaxone-resistance plasmid

Selective culturing and *sipB*–*C* PCR confirmed the identity of the *Salmonella* (results not shown) and MICs confirmed ceftriaxone resistance (Fig. 2). PCR using the β -lactamase consensus primers yielded a product of the expected size, 1.1 kb, identified as *bla*_{CMY-2} by sequence analyses (Fig. 2). BLAST

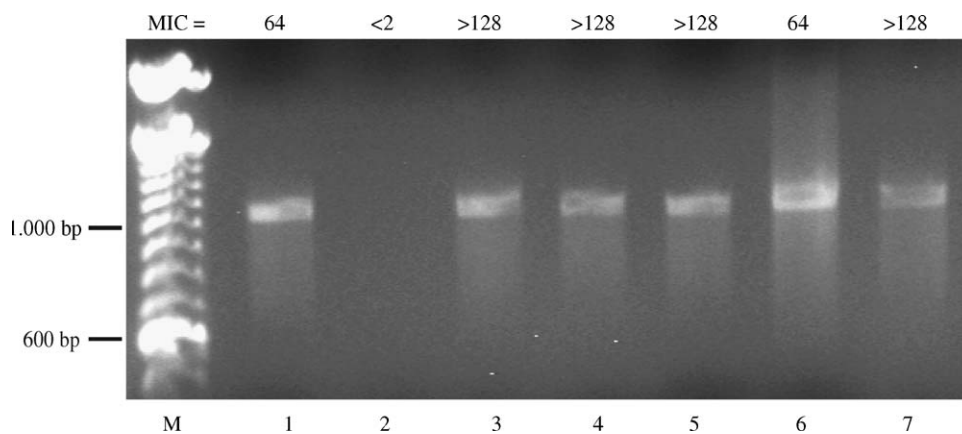


Fig. 2. Verification of gene transfer using PCR of the β -lactamase gene (1.1 kb) and MICs for ceftriaxone. MICs are reported above the lanes as $\mu\text{g/ml}$ (breakpoint = $32 \mu\text{g/ml}$). Lane 1, *Klebsiella pneumoniae* TCR2003; lane 2, native *Salmonella* Typhimurium SL1344; lane 3, in vivo goat isolate of SL1344; lane 4, in vivo calf isolate of SL1344; lane 5, in vivo sheep isolate of SL1344; lane 6, in vitro bovine RPz isolate of SL1344; lane 7, in vitro caprine RPz isolate of SL1344.

analysis showed this sequence was identical to the published sequence for *K. pneumoniae* (Bauernfeind et al., 1996). Using primer walking, sequence data was obtained for regions flanking this gene and BLAST searches showed 100% identity to several plasmids that had been previously characterized from *Salmonella* (Giles et al., 2004). Two of these plasmids carried a unique region that BLAST searches did not find in any other plasmids. One of these plasmids had an IS26 sequence. PCRs using primers for both the unique region and IS26 were positive, thus suggesting that plasmid “C” (Giles et al., 2004) was present in *K. pneumoniae* TCR2003.

4. Discussion

Herein, a site for the transfer of antibiotic resistance between bacteria was demonstrated and factors governing this phenomenon, both in vitro and in vivo, were evaluated. Existence within RPz, as well as within macrophages that likely mimic some functions of RPz, apparently induces the donor *Klebsiella* to conjugate with an *S. Typhimurium* recipient. The plasmid transferred to the recipient bears a β -lactamase gene, *bla*_{CMY-2}, that confers ceftriaxone resistance. Subsequent bovine in vivo studies indicated that gene transfer to SL1344 can also occur with other donors, i.e., TME2003, and other plasmids. These data indicate that RPz are a contributing factor in bacterial gene transfer.

Genetic exchange in the rumen appears to be directly related to the differential survival of bacteria upon protozoal digestion. Typically, bacteria ingested by RPz are sequestered in vacuoles for digestion (Coleman and Hall, 1969). While the survival of *Salmonella* in RPz has yet to be explicitly examined, studies have shown the growth of *Salmonella* in contractile vacuoles in amoeba (Gaze et al., 2003; Tezcan-Merdol et al., 2004). *Klebsiella*, however, appears to be incapable of long-term survival within protozoa (unpublished observations). Thus, the transfer event may be dependent upon a protozoa-insensitive recipient and a protozoa-sensitive donor. The same situation could occur in macrophages although it is unlikely that *Salmonella* and *Klebsiella* would be sufficiently coexisting in macrophages in vivo. That is, the phenomenon reported herein is likely

a result of the combination of RPz-induced stress conditions and intra-RPz “crowding” of bacteria that decreases inter-bacterial proximities. The high concentration of bacteria eventually reaches a threshold in which the transfer event occurs. This threshold is unlikely to be broached in macrophages.

Conjugation appears to be the route for the transfer event between TCR2003 and SL1344 for four reasons. First, to date, there are no reports of natural transformation (i.e., acquisition of naked DNA) in *Salmonella*. In fact, artificially competent *Salmonella* were incapable of plasmid uptake (data not shown). Second, the ceftriaxone-resistance plasmid is a conjugative plasmid (Carattoli et al., 2002; Giles et al., 2004). Third, intra-protozoal conjugation has been previously demonstrated for other bacteria (Schlimme et al., 1997). Fourth, nalidixic acid, a conjugation inhibitor, prevented the transfer of the ceftriaxone-resistance plasmid into a nalidixic acid-resistant recipient *Salmonella*. While other avenues of transfer were not investigated, e.g., bacteriophage-mediated transduction, the phenomenon represents a major public health problem regardless of the mechanistic basis.

While our study is the first to document RPz-mediated conjugation, native conjugation is also contributing to gene transfer since turkey poult intestinal tracts were recently reported as sites of gene transfer from *E. coli* to *Salmonella* (Poppe et al., 2005). That is, the absence of protozoa in the avian intestinal tract suggests a native transfer event. Given the large volume of the rumen, however, the phenomenon reported herein could be as important.

In another phase of our study, we document the exchange of a colicin-encoding plasmid which represents yet another way for bacteria to disseminate selective advantages. While the acquisition of antibiotic resistance genes is more important for public health, the acquisition of colicin production could precipitate focal disease outbreaks. For example, a pathogen could temporarily disrupt the overall gut ecology if it can acquire a colicin that kills some of the commensal bacteria that maintain intestinal homeostasis. The resulting dramatic change in the intestinal flora community could allow for the rapid expansion, i.e., onset of disease, and shedding of the pathogen. Therefore, acquisition of colicin production could indirectly lead to an apparent precipitation of

virulence. However, the “rock-paper-scissors” paradigm for colicin-producing strains suggests that the microfloral population profile would eventually return to normal homeostatic levels (Kirkup and Riley, 2004).

While we did not characterize the colicin-encoding plasmid, sequence data indicates the plasmid carried by the TCR2003 environmental isolate has previously been described from the Newport and Typhimurium serotypes of *S. enterica* (Carattoli et al., 2002; Giles et al., 2004). Both of these studies attempted in vitro conjugation experiments with this plasmid but were unsuccessful which is consistent with the absence of transfer observed in our studies under typical conjugation conditions. Notably, the β -lactamase sequence was identical to a previously published sequence for a plasmidic *bla*_{CMY-2} gene from *K. pneumoniae* (Bauernfeind et al., 1996). Clearly, this is a mobile plasmid and its distribution in nature is likely greater than what is recorded in the literature. Because of the therapeutic significance of third-generation cephalosporins, the apparent promiscuity of this plasmid deserves additional consideration.

Our study also shows that defaunation may prove to be an important method to reduce the spread of antibiotic resistance genes. Without the RPz predation, the change in the rumen bacterial community composition will bear significance on the relative success of pathogens in the rumen, as well as selective pressures for antibiotic resistance. However, previous studies on the success and sustainability of defaunation are conflicting and long-term research is lacking. Some reports have indicated no short-term adverse effects on animal health following defaunation (Ankrah et al., 1990; Orpin, 1977) yet others have demonstrated detriment to the animal (Lovell et al., 1982; Moffatt et al., 1975). Notably, such ill effects appear to be due to the defaunation agent rather than the actual defaunation. While a plethora of research suggests that defaunation may actually increase growth in ruminants fed protein-limited diets, no data is available for the long-term effects (Williams and Coleman, 1992, 1997). Continued efforts in evaluating the efficacy and benefits of various defaunation schemes are necessary for determining the utility of this strategy in ruminant livestock management.

In summary, these studies provide the first documentation of the role of RPz in bacterial gene

transfer. Notably, the ability to survive protozoa engulfment, as seen with *Salmonella* strains SL1344 and χ 4232, is requisite for this phenomenon to occur. Future work aimed at developing sustainable defaunation may aid attempts to curtail the spread of antibiotic resistance in bacteria and dictate antibiotic therapies in veterinary medicine. It should be noted, however, that this conjugation event could occur elsewhere (e.g., the cecum) if protozoa are present.

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